REMARKS

The Office Action dated May 30, 2006 has been reviewed and the comments of the U.S. Patent and Trademark Office have been considered. The following remarks are respectfully submitted to place the application in condition for allowance.

A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier. Claims 1-33 were previously canceled. Claims 35-87 are presently canceled. Claim 34 has been amended. Support for the amendment may be found, for example, at least in the following sections of the original disclosure: page 7, lines 5-6; page 8, lines 21-22; page 11, line 28 - page 14 line 9; page 21, line 26 - page 22, line 21; and page 23, lines 9-15. New claims 88-132 have been added. Support for the new claims may be found, for example, in at least the following sections of the original disclosure: page 6, lines 20-22; page 7, lines 18-19 and 22-28; page 8 lines 6-7 and 18-20; page 12, lines 1-4; page 16, line 30; page 17, lines 19-22; page 18, line 13; page 19, lines 24-29; page 23, line 11, page 24, lines 23-24. Claims 34 and 88-132 are currently pending in this application.

Information Disclosure Statement

The Examiner states that the Information Disclosure Statement filed July 28, 2004 fails to comply with 37 CFR 1.98(a)(3) because the reference EP 0560156 did not include a concise explanation of the content of the information. Applicants hereby submit an English translation of the abstract of EP 0560156.

The Examiner also states that the two lists submitted containing the references relating to the pending litigation of patent 6,573,099 have not been considered because they are not proper information disclosure statements. These lists were submitted by an adverse party during the

litigation of the previously mentioned patent. Applicants are submitting these lists in order to completely comply with their ethical duty to disclose all information to the US Patent and Trademark Office. Applicants point out that the lists consist of the full and complete set of material made known to Applicants as submitted by the aforesaid adverse party in the litigation.

Rejections Under 35 USC §112

Claims 34-51 and 70-87 are rejected under 35 USC §112, first paragraph as failing to comply with the written description requirement. The Examiner states that the specification does not describe expression constructs comprising sequences with less than 100% identity with a target gene, nor does it describe a construct comprising structural gene sequences of any degree of identity that meet the size limitation of 20-30 nucleotides.

Applicants respectfully traverse the rejection. However, to expedite prosecution of the present application, claims 35-51 and 70-87 have been canceled, thus making the rejection with respect to those claims moot. Claim 34 has been amended to recite a sequence that is identical to "30 contiguous nucleotides" of the target gene. These limitations are described in the specification as filed as indicated above, specifically on page 8, lines 21-22. Withdrawal of the rejection is respectfully requested.

Claims 40-42, 44-47, 76-78 and 80-83 are rejected under 35 USC §112, first paragraph as failing to comply with the written description requirement. The Examiner states that the claims were presented in a preliminary amendment and are considered new matter because they are not fully supported in the original disclosure. The Examiner contends that the specification does not contemplate use of structural gene sequences with the exact lengths recited in the claims and that the specification is silent with regard to the relative lengths of the two copies a structural gene sequence.

Applicants respectfully disagree with the Examiner's rejection. However, in the interest of advancing prosecution of this case, claims 40-42, 44-47, 76-78 and 80-83 have been canceled in the present application, thus making the rejection in respect to those claims moot. Applicants therefore respectfully request withdrawal of the rejection. In addition, amended claim 34 and new claims 88-131 recite a nucleotide sequence which is identical to a sequence of 30 contiguous nucleotides. Support for this length is found in the specification as indicated above. Applicants respectfully request withdrawal of the rejection.

Claims 34-51 and 70-87 are rejected under 35 USC §112, first paragraph. The Examiner states that while the specification is enabling for a method of reducing expression of a target gene in an animal cell *in vitro*, it is not enabling for a method of reducing expression of a target gene in an animal cell *in vivo* in any organism. The Examiner further contends that the specification does not provide any working examples of the use of the genetic constructs for the inhibition of gene expression in any animal cell *in vivo* or *in vitro*.

Applicants respectfully disagree with the rejection. However, to further prosecution, Claims 35-51 and 70-87 have been cancelled, thus making the rejection with respect to those claims moot. As acknowledged by the Examiner on page 6 of the Office Action dated May 30, 2006, the claimed method is enabled for animal cells *in vitro*. Claim 34 has been amended to recite a method for producing an RNA molecule for reducing expression of a target gene in an isolated eukaryotic cell, consistent with the Examiner's acknowledgement. New claim 114 is directed towards a method for producing an RNA molecule for reducing expression of a target gene in plant cell. It is respectfully submitted that the skilled person, on being taught the structure of the synthetic genetic construct as described in the specification and as recited in the claims as amended, is readily able to make and use the construct in an isolated eukaryotic cell or

in a plant cell to produce the RNA molecule as claimed. Withdrawal of the rejection is respectfully requested.

Rejections Under 35 USC §103(a)

Claims 34-51 and 70-87 are rejected under 35 USC §103(a) as being obvious over Fire *et al.*, as evidenced by Zhang *et al.*, and in view of Jendrisak *et al.*, Noonberg *et al.* and Florkiewicz *et al.* The Examiner contends that Fire *et al.* teach a method of inhibiting gene expression in cells using double-stranded RNA comprising a sequence complementary to the target gene and a sequence identical to the target gene. Further, the Examiner states that the post-filing of Zhang *et al.* indicate that the Dicer ribonuclease would provide nucleotide sequences of the lengths claimed in the present invention. In the Examiner's view, Noonberg *et al.*, Jendrisak *et al.* and Florkiewicz *et al.* provide complementary teachings regarding expression of nucleic acids. The Examiner then concludes that it would have been obvious to utilize the method of Fire *et al.* to make the vectors as taught by Noonberg *et al.*, Jendrisak *et al.* and Florkiewicz. The motivation to combine the references, as argued by the Examiner, is provided because Fire *et al.* suggest double-stranded RNA may be produced by a vector, Noonberg *et al.* teach that vectors may comprise a promoter, a sequence to be transcribed and a terminator, Jendrisak *et al.* teach that using multiple promoters produce RNA more efficiently, and Florkiewicz teaches vectors to express proteins. Finally, the Examiner contends that one would have a reasonable expectation of success in combining the references because Fire *et al.* teach that double-stranded RNA inhibits gene expression and the remainder of the references teach that production of RNA from vectors is routine in the art.

Applicants respectfully disagree that the present claims are obvious over the cited references. Claims 35-51 and 70-87 have been cancelled, thus making the rejection with respect to these claims moot.

In levying an obviousness rejection under 35 U.S.C. §103, the Examiner has the burden of establishing that at the time of the invention, there was: (1) some suggestion or motivation to modify the reference or to combine reference teachings, (2) a reasonable expectation of success, and (3) that the prior art references, when combined, taught or suggested all the claim limitations. See MPEP §2143 (Aug. 2001, Latest Revision August 2005); *See also In re Royka*, 490 F.2d 981, 985 (C.C.P.A. 1974). Obviousness may not be established based upon hindsight or the teachings or suggestions of the inventor. *W. L. Gore & Assocs., Inc. v. Garlock, Inc.*, 721 F.2d. 1540, 1551, 1553 (Fed. Cir. 1983); *Ruiz v. A. B. Chance Co.*, 357 F.3d 1270, 1276 (Fed. Cir. 2004). Here, the Examiner has not met this burden. It is respectfully submitted that the obviousness rejection fails on each of the three grounds, as detailed below.

Moreover, the Examiner appears to be using Zhang *et al.* to suggest that the product inherently would be that which is required by the claims. While Applicants are familiar with "inherent anticipation", Applicants are not aware of any case from the Federal Circuit or its predecessor courts that adopted the concept of "inherent obviousness", which the Examiner appears to be arguing.

Claim 34 has been amended and new claim 114 added to recite a method for producing an RNA molecule cell as a transcript of a synthetic genetic construct having a particular configuration of elements. The method comprises introducing into the cell a genetic construct comprising two copies of a structural gene sequence, each of 30 nucleotides. The two copies are under the control of a single promoter and terminator sequence and are spatially separated by a stuffer fragment comprising a sequence of nucleotides. Fire *et al.* neither teach nor contemplate

the use of a stuffer fragment to spatially separate two copies of the structural gene. Moreover, Fire *et al.* do not disclose the length limitation of 30 nucleotides, or the combination of the two copies each having 30 nucleotides, or the combination of all of these elements in the order as claimed.

The deficiencies of Fire *et al.* are not cured by the additional references. Jendrisak *et al.* teaches a vector for the *in vitro* production of RNA copies of either strand of a cloned DNA sequence, not production of double-stranded RNA in eukaryotic cells as presently claimed. Jendrisak *et al.* do not utilize or suggest a stuffer fragment, let alone a stuffer fragment between two copies of the structural gene sequence, the two copies being operably linked and in opposite orientation with respect to a single promoter. The construct of Jendrisak *et al.*, in contrast to Applicants' presently claimed invention utilize two different opposed promoter sequences and only a single structural gene sequence. See Abstract; Col. 2, ll. 25-27Col. 3, ll. 56-58. Noonberg *et al.* teach methods and constructs for the delivery of ribozyme oligonucleotides intracellularly. The RNA polymerase III-based constructs, termed "oligonucleotide generators", do not contain the stuffer fragments of Applicants' present invention, or two copies of the structural gene sequence, each of 30 nucleotides, even less the combination of elements as presently claimed. Florkiewicz contemplates protein expression vectors, not vectors for expression of double-stranded RNA. Florkiewicz neither contemplates nor teaches the stuffer fragment, the length limitation of the structural gene sequence, the multiple copies of the structural gene sequences or any combination of these elements, let alone the particular arrangement of elements of the presently claimed invention. Insofar as any of these secondary references is of any relevance (which is vigorously contested), they in fact teach away from the presently claimed subject matter. For example, Jendrisak *et al.*, teach the use of one sequence to

be transcribed with two opposing promoters, leading the skilled person in the opposite direction to the presently claimed invention.

The PTO defines one of ordinary skill in the art as one "who thinks along the lines of conventional wisdom in the art and is not one who undertakes to innovate, whether by patient, and often expensive, systemic research or by extraordinary insights, it makes no difference which." *Ex parte* Anderson, 21 U.S.P.Q.2d. 1241, 1256 (B.P.A.I 1991). For the reasons set forth above, an artisan thinking along the line of conventional wisdom would not have been motivated to modify the Fire *et al.* reference with Jendrisak *et al.*, Noonberg *et al.* and Florkiewicz *et al.* as the Examiner suggests, nor would an artisan of ordinary skill have a reasonable expectation of success.

Thus, even if Fire *et al.* was analogous art and combinable with Jendrisak *et al.*, Noonberg *et al.* and Florkiewicz *et al.* (which Applicants dispute), the secondary references do not cure the Fire *et al.* deficiencies because the combination does not teach each and every element of Applicants' present invention, let alone the combination of elements.

Applicants further point out that the dependent claims of the present application contain further limitations that are not disclosed nor suggested by the prior art.

Moreover, there is not teaching nor suggestion in the prior art that the presently claimed invention provides advantages in producing double-stranded RNA in a eukaryotic cell for reducing expression of a target gene. In particular, references such as Levin *et al.*, 2000 and Smith, *et al.* 2000 (see attached) confirm the advantageous properties of the synthetic genetic constructs comprising the stuffer fragment.

Applicants respectfully request withdrawal of the rejection.

In view of the above amendment, applicant believes the pending application is in condition for allowance.

Applicants submit concurrently a request for extension of time under 37 C.F.R. 1.136 and the accompanying fee. In the event that any additional extensions of time are necessary to prevent the abandonment of this patent application, then such extension of time are hereby petitioned. The U.S. Patent and Trademark Office is hereby authorized to charge any fees that may be required in conjunction with this submission to Deposit Account Number 50-2228, referencing matter number 023004.0104N4US.

Dated: November 30, 2006

Respectfully submitted,

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Pseudorabies virus polynucleotids and their use in the production of virus-resistant eucaryotic cells.

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Publication date: 1993-09-15

Inventor: SPRINGER WOLFGANG DR (DE); BAUMGARTEN JOERG DR (DE); KRETSCHMER AXWL DR (DE); KOELBL HEINZ DR (DE); LOEBBERDING ANTONIUS DR (DE); STRUBE WALTER DR (DE); THEIN PETER PROF DR (DE)

Applicant: BAYER AG (DE)

Classification:

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- AU662040B (B2)

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Abstract of EP0560156

The invention relates to polynucleotides which contain pseudorabies virus sequences, especially sequences from the gII region of PRV, in the antisense orientation, to their preparation and to their use.

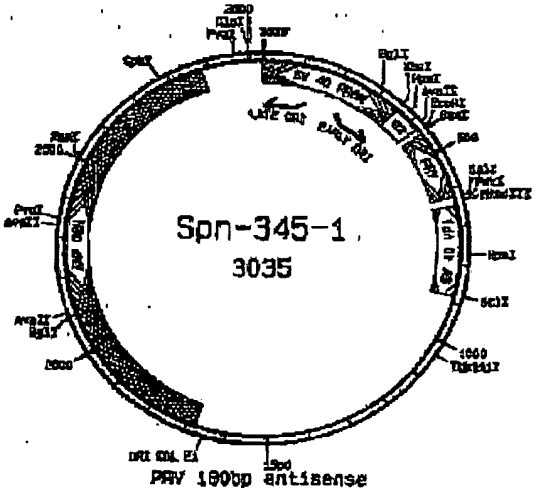

FIG.2

Plant Molecular Biology 44: 759–775, 2000.

Methods of double-stranded RNA-mediated gene inactivation in *Arabidopsis* and their use to define an essential gene in methionine biosynthesis

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Abstract

Controlled down-regulation of endogenous plant gene expression is a useful tool, but antisense and sense silencing lack predictability. Recent studies show that expression of both antisense and sense RNA together is an effective means of inactivating reporter and viral genes in plants. We created transgenic plants expressing antisense and sense RNA together in a single 'double-stranded RNA' (dsRNA) transcript. This approach shows great promise as a highly effective means for reducing gene function. With this approach, we demonstrated that the *Arabidopsis* cystathionine β-lyase gene, which encodes a methionine biosynthetic enzyme, is essential for viability. Inactivation of this gene was rescued by the addition of methionine to the growth medium. Compared to antisense and sense constructs, the dsRNA construct showed a much more consistent and complete suppression of gene activity. Additionally, expression of a transcript with a spacer sequence containing an unrelated gene between antisense and sense luciferase gene fragments led to stronger inactivation of a second luciferase transgene than did constructs with a minimal spacing between sense and antisense fragments. However, the gene in the spacer region was neither functionally expressed nor functional in silencing a second, unlinked homologous transgene.

Abbreviations: dsRNA, double-stranded RNA; PTGS, post-transcriptional gene silencing; TGS, transcriptional gene silencing; GUS, β-glucuronidase (GUS); CGS, cystathionine γ-synthase; CBL, cystathionine β-lyase; *ACT2*, *ACTIN2*; RLU, relative light unit; ssRNA, single-stranded RNA; AVG, L-aminoethoxyvinylglycine; PAG, propargylglycine

Introduction

To answer basic questions relating to gene function and for the beneficial manipulation of metabolism or other cellular processes in transgenic plants, it is important to have a reproducible and effective method of down-regulation of endogenous plant genes. Commonly available methods, such as antisense, sense co-suppression (reviewed in Bruening, 1998), ribozymes (Merlo *et al.*, 1998), or chimeric oligonucleotides (Zhu *et al.*, 1999) do not yet yield consistent and predictable results or are technically challenging. Recently it has been shown that the simultaneous expression of both antisense and sense gene fragments provides a more reliable method of inactivation of viral and reporter genes (Waterhouse *et al.*, 1998). In this paper, we seek to extend these results to an endogenous plant gene and to explore the mechanism of gene silencing.

Homology-dependent gene silencing in plants has been observed by many researchers, yet this phenomenon remains incompletely understood (reviewed in Vaucheret *et al.*, 1998; Grant, 1999; Kooter *et al.*, 1999; Waterhouse *et al.*, 1999). Silencing can be triggered by the introduction of a transgene and can lead to loss of expression of both the transgene and the

homologous endogenous genes. Post-transcriptional gene silencing (PTGS) has been distinguished from transcriptional gene silencing (TGS) by the observation of continued transcription in the former. Although the mechanism of PTGS remains uncertain, it has been suggested that small antisense RNAs may be formed by RNA-dependent RNA polymerases (Schiebel *et al.*, 1998; Cogoni and Macino, 1999) and that such RNAs could constitute the specificity determinant of PTGS (Hamilton and Baulcombe, 1999). Changes in the methylation state of both TGS and PTGS silenced genes have also been observed, but their significance remains to be determined (Wassenegger and Pelissier, 1998; Kooter *et al.*, 1999). While PTGS can be an unintended result in the creation of transgenic plants over-expressing a gene of interest or a useful tool for the determination of gene function, it may benefit plants by providing protection from viruses and deleterious transposition events.

How is silencing induced? It has been shown that constructs designed to express either sense or antisense RNA for a gene of interest can lead to gene silencing (reviewed in Bruening, 1998). Recent research in plants, animals, and fungi suggests that combining sense and antisense RNA to produce double-stranded RNA (dsRNA) is more effective in gene silencing. This advance was first reported for *Caenorhabditis elegans*, in which injecting or feeding exogenous dsRNA leads to PTGS of homologous sequences (Fire *et al.*, 1998). Tobacco containing one transgenic construct designed to produce antisense transcripts for the viral PVY PRO gene and a second construct to produce sense transcripts showed greater resistance to PVY infection than tobacco containing only one of the two constructs (Waterhouse *et al.*, 1998). In addition, a construct expressing both sense and antisense β-glucuronidase (GUS) sequences in a single RNA transcript showed better silencing of a second GUS transgene in rice than either sense or antisense alone (Waterhouse *et al.*, 1998). Finally, a construct expressing an inverted repeat of the 5′-UTR of the tomato *ACO1* gene together with the rest of the *ACO1* cDNA in a sense orientation showed improved silencing compared to a simple sense *ACO1* construct (Hamilton *et al.*, 1998). These data suggest that antisense and sense PTGS may share a common mechanism and that they both lead to the production of dsRNA, perhaps through the generation of undetected levels of the complementary RNA. Alternatively, they may not share a common mechanism, but combining the two can lead to a synergistic increase in PTGS.

With these developments in the field of gene silencing, we sought to apply them to examine the effect of the inactivation of a gene in the methionine biosynthetic pathway. This pathway is of interest as methionine is a precursor not only for protein synthesis, but also for the production of polyamines, the hormone ethylene, and *S*-adenosylmethionine, a compound important for transmethylation reactions (reviewed in Ravanel *et al.*, 1998). From an applied science point of view, a better understanding of this pathway, which is found in plants but not animals, might lead to the development of plants with improved nutritional properties or novel herbicides. The first committed step of this pathway is carried out by cystathionine γ-synthase (CGS; EC 4.2.99.9), which catalyzes the conversion of *O*-phosphohomoserine and cysteine to cystathionine. The second step is carried out by cystathionine β-lyase (CBL; EC 4.4.1.8), which catalyzes the conversion of cystathionine to homocysteine, pyruvate, and ammonia. The final step is carried out by cobalamin-independent methionine synthase (EC 2.1.1.14), which catalyzes the transfer of a methyl group to homocysteine from N5-methyltetrahydrofolate. *Arabidopsis* homozygous for an antisense CGS transgene exhibited yellowing and seedling lethality and CGS activity was reduced below about 20% of wild-type levels (Ravanel *et al.*, 1998). The sequence of the *Arabidopsis* CBL gene has been determined (Ravanel *et al.*, 1995; Bork and Hell, 1997) and we undertook experiments by a 'dsRNA' approach to determine the phenotype of plants with decreased levels of CBL. In this paper, we use the term 'dsRNA' for constructs expressing both antisense and sense RNA, with the caveat that there is no direct observation of the formation of dsRNA *in vivo*. Using a dsRNA approach, we show that CBL is an essential gene. A comparable approach using only antisense or sense constructs does not provide a great enough reduction in CBL function to reach this conclusion.

In this paper, we significantly extend findings in the use of dsRNA constructs to induce PTGS in three ways. First, we demonstrate that an essential endogenous plant gene can be silenced with a dsRNA transgene. Second, we show that there is no detectable silencing caused by a gene placed in the loop region between the sense and antisense stem of a second gene. Third, we provide data showing the comparative extent of silencing at the RNA and protein activity levels.

Materials and methods

Molecular biology

Total RNA was isolated with the RNeasy Plant Mini kit (Qiagen, Valencia, CA) or as per Lagrimini (1987) for CBL experiments and as per Verwoerd (1989) for luciferase experiments. RNA gel blots were prepared and hybridized as described (Guyer *et al.*, 1998). A DNA product corresponding to a 3′ portion of the CBL cDNA that was not included in the transgenic constructs was prepared by PCR with primers CBL9 (5′-ATGCAAGCATACCTGCAGAA-3′) and CBL10 (5′-AAGCTGAGACAAGAACAAACCA-3′) and the pFL61 *Arabidopsis* cDNA library (Minet *et al.*, 1992) as a template. For RNA gel blots, a ^{32}P-labeled CBL probe was prepared by PCR using the same primers and this PCR product. For RNA gel blots, a ^{32}P-labeled *ACTIN2* (*ACT2*) probe was prepared using the Random Primers DNA Labeling System (Life Technologies, Gaithersburg, MD) with a gel-purified fragment from EST 168H2T7 (GenBank R65270) (Newman *et al.*, 1994).

Plasmid construction

In each CBL construct, the CBL gene fragment used corresponds to nucleotides 13 to 1159 of the cDNA (GenBank L40511; Ravanel *et al.*, 1995; Figure 1). In the CBL cDNA, the ATG start codon is located at 117–119 and the stop codon is located at 1509–1511. For the CBL antisense construct, plasmid pJG304/aCBL was made by digesting pJG304 ΔXhoI (Guyer *et al.*, 1998) with *Nco*I and *Sac*I to excise the GUS gene. The GUS gene was replaced with a *Nco*I- and *Sac*I-digested CBL PCR product, which was generated using primers DG354 (5′-GATCGAGCTCCACGAGAACTGTCTCCG-3′) and DG357 (5′-TCAGCCATGGGAAGACAAGTACATTGC-3′) and the pFL61 cDNA library. pJG304/aCBL was cut with *Xho*I to excise the cassette containing the GAL4 DNA-binding site/35S minimal promoter/antisense CBL/CaMV terminator fusion. This cassette was ligated into *Xho*I-digested pJG261 binary vector (Guyer *et al.*, 1998), producing pJG261/aCBL. For the CBL sense construct, pJG304/sCBL was made by digesting both pJG304ΔXhoI and a CBL PCR product with *Nco*I and *Sac*I and ligating them together. This product was generated with primers CBL1 (5′-CTTGCCATGGCACGAGAACTGTCTCCG-3′) and CBL2 (5′-CATGGAGCTCGAAGACAAGTACATTGCA-3′) and the pFL61 cDNA library. pJG304/sCBL was cut with *Xho*I and ligated to *Xho*I-digested pJG261, producing pJG261/sCBL. For the dsRNA CBL construct, pJG304/dsCBL was made by digesting both pJG304/aCBL and a CBL PCR product with *Sac*I. This product, which was generated with CBL2 and CBL3 (5′-CATCGAGCTCCTCTGTTTAAACCACGAGAACTGTCTCCGTCGC-3′) and the pFL61 cDNA library, was inserted in the sense orientation downstream of the antisense CBL sequence. *Escherichia coli* SURE2 (Stratagene, La Jolla, CA) was used as the bacterial host to stabilize this construct. pJG261/dsCBL was made from pJG304/dsCBL cut with *Xba*I and ligated to *Spe*I-digested pJG261, producing pJG261/dsCBL. *E. coli* XL1-BLUE MRF′ (Stratagene) was used as the bacterial host to partially stabilize the construct. Unrearranged DNA for this construct was isolated by agarose gel purification.

Two binary constructs, pAdF2 and pAdF4, which were designed to produce a dsRNA transcript, were prepared by cloning both the sense and antisense orientations of a 3′ portion of the luciferase gene (Figure 1). A 670 bp 'sense'-oriented fragment of the firefly luciferase gene was prepared by PCR with TurboPfu (Stratagene), primers ds-Luc1 (5′-CGCGGATCCAAGATTCAAAGTGCGCTGCTG-3′; *Bam*HI site underlined) and ds-Luc2 (5′-GCGAAGCTTGGCGACGTAATCCACGATCTC-3′; *Hin*dIII site underlined), and plasmid pGL3 (Promega, GenBank U47295) as a template. A 669 bp 'antisense'-oriented fragment of the luciferase gene was prepared by PCR with primers ds-Luc3 (5′-CGGTCTAGAAAGATTCAAAGTGCGCTGCTG-3′; *Xba*I site underlined) and ds-Luc2 and pGL3 as a template. The resulting 'sense' and 'antisense' DNA fragments were digested with *Bam*HI and *Hin*dIII or *Xba*I and *Hin*dIII, respectively. Construct pAdF3 was created by a three-way ligation with these two fragments and *Bam*HI- and *Xba*I-digested pLITMUS 28 (New England Biolabs). A 563 bp DNA fragment containing the *bar* gene open reading frame (D'Halluin *et al.*, 1992) was prepared by PCR with primers bar-1 (5′-GCGAAGCTTGATCCATGAGCCCAGAACGA-3′; *Hin*dIII site underlined) and bar-2 (5′-GCCAAGCTTCCTAGAACGCGTGATCTC-3′; *Hin*dIII site underlined), and plasmid pCSA104 (D. Patton, personal communication) as a template. pAdF1 was generated by cloning the *Hin*dIII-digested *bar* PCR product into the unique *Hin*dIII site of pAdF3. In pAdF1, the *bar* gene open reading frame is in the same orientation as that of the 'sense' luciferase fragment. pAdF4 was created by a three-way ligation with the *Bam*HI-*Sac*I

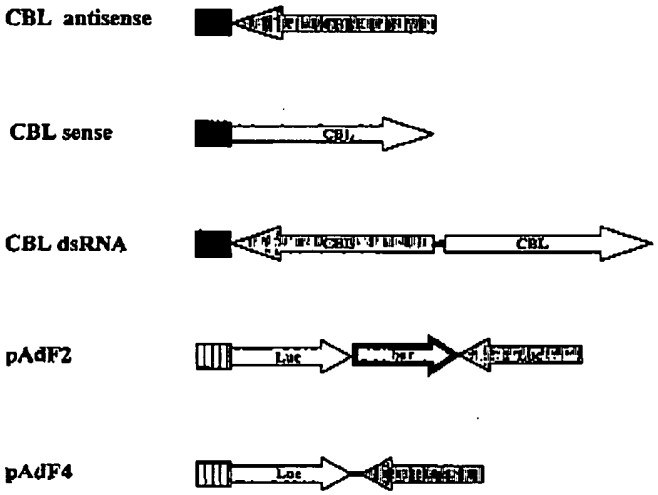

Figure 1. Constructs used in this study. Promoters are represented by rectangles at the left of each construct: a filled rectangle for the UAS GAL4 promoter (see Materials and methods) and a rectangle with vertical stripes for the ACT2 promoter. Gene fragments in the sense orientation are represented by white arrows pointing to the right. Gene fragments in the antisense orientation are represented by gray arrows pointing to the left. DNA sequences inserted between the sense and antisense sections of a gene are represented either with a thick black line for the 18 bp in the CBL dsRNA construct and 6 bp in the pAdF4 construct, or a thick black arrow for the *bar* gene in the pAdF2 construct. These three spacer sequences are not drawn to scale.

fragment from pAdF3, which consists of both sense and antisense luciferase fragments, a 1.2 kb *Bam*HI-*Spe*I DNA fragment containing the *Arabidopsis ACT2* promoter (An *et al.*, 1996), and binary plasmid pSGCHC1 digested with *Spe*I and *Sac*I. pSGCHC1, which is a derivative of pGPTV-HPT with the GUS gene replaced by the polylinker from pBluescript KS+ (Becker *et al.*, 1992), carries a kanamycin resistance gene for bacterial selection and a hygromycin resistance gene for transgenic plant selection (S. Goff, personal communication). pAdF2 was constructed with a cloning strategy similar to that used for pAdF4, except that a fragment from plasmid pAdF1, which contains the *bar* gene in between the sense and antisense luciferase gene fragments, was used in place of the pAdF3 fragment.

CBL transgenic plants

The three described pJG261/CBL constructs were electro-transformed (BioRad, Hercules, CA) into *Agrobacterium tumefaciens recA*$^-$ strain (Lazo *et al.*, 1991), and *Arabidopsis* Col-0 plants were transformed by infiltration (Bechtold *et al.*, 1993). Prior to transformation of *Arabidopsis*, plasmids were checked by restriction digest and PCR for the presence of DNA that had not rearranged. For pJG261/aCBL and pJG261/sCBL, all DNA appeared intact; for pJG261/dsCBL, ca. 35% of the DNA appeared intact (data not shown). For pJG261/aCBL, an additional experiment was done with the *Agrobacterium* GV3101 (pMP90) strain (Koncz and Schell, 1986). Seeds from the infiltrated plants were selected on germination medium containing 15 μg/ml Basta (Guyer *et al.*, 1998).

Transgenic plants containing a GAL4-binding site/minimal CaMV 35S promoter/CBL construct were transplanted to soil and grown to maturity in the greenhouse. The presence of a transgenic CBL fragment in each line was confirmed by PCR. For the antisense construct, primers ASV1 (5′-TTTGGAGAGGACAGACCTGC-3′) and CBL3 were used to verify the presence of a ca. 1200 bp product. For the sense construct, primers ASV2 (5′-GGATTTTGGTTTTAGGAATTAGAA-3′) and CBL3 were used to verify the presence of a ca. 1200 bp product. For the dsRNA construct, primer pairs of ASV2 with CBL3 and of ASV1 with CBL3 were used to verify the presence of a ca. 1200 bp product with each pair. Each of the primary transgenic CBL transformants was pollinated by the homozygous GAL4/C1 transactivator line pAT53-103 (Guyer *et al.*, 1998).

About 25 F_1 seeds per line were plated on MS + 2% sucrose medium (4.3 mg/ml Murashige-Skoog salts, 0.5 mg/ml MES, 20 mg/ml sucrose) and grown in a growth room at 20 to 22 °C with a day length of 13 h. For sense lines 2-20 and 2-23, the five healthiest (based on size and color) affected seedlings were transplanted to soil. For dsRNA lines 3-15, 3-18, and 3-21, the six healthiest affected seedlings were transplanted to soil. For other lines with only wild-type F_1 phenotypes, eight seedlings were transplanted to soil. F_1 plants were then grown in the greenhouse at 18 to 23 °C with a day length of 14.5 h. The presence of the CBL transgene in F_1 plants with a wild-type phenotype was assessed by PCR.

For methionine rescue experiments, 12 F_1 seeds from lines 3-15, 3-18, and Col-0 were plated on MS + 2% sucrose supplemented with 0, 150, or 250 μM L-methionine. After 13 days in the growth room, six Col-0 and twelve 3-15 F_1 plants were transplanted from 250 μM methionine plates to soil and placed in the greenhouse.

Luciferase transgenic plants

Plasmids pAdF2 and pAdF4 were introduced into *Agrobacterium tumefaciens* strains GV3101 and AGL1, and both *Arabidopsis* Col-0 and transgenic line pPH108 plants were transformed by infiltration (Bechtold *et al.*, 1993). This kanamycin-resistant pPH108 transgenic line expresses very high levels of luciferase due to the presence of the firefly luciferase reporter gene (from pGL3; Promega) under the control of the *Arabidopsis UBQ3* promoter (Norris *et al.*, 1993) followed by a fusion of the *UBQ3* and CaMV 35S 5′-UTRs (P.B. Heifetz, unpublished observations). This line was hemizygous for the pPH108 luciferase-expressing transgene. No plasmid DNA rearrangements were detected by restriction digest analysis in any of the *Agrobacterium* strains used for the plant transformations. T_1 seeds from the infiltrated plants were germinated on medium containing 25 μg/ml hygromycin B alone or in combination with 50 μg/ml kanamycin monosulfate for selection of transgenic Col-0 or for supertransformed pPH108, respectively. These seedlings were transferred to soil and grown in phytotron-controlled conditions.

TaqMan assays

For CBL, primers CBL7 (5′-AGCATCTCGTAGAAAC CACCAAA-3′) and CBL8 (5′-GCATGGCATGCTTAT CAGTGA-3′) were used with a CBL probe (FAM (6-carboxyfluorescein) 5′-AACACTCCCAAAACTGA-CAGCTATGCTGAAGT-3′ TAMRA (6-carboxy-N,N, N′,N′-tetramethylrhodamine)) in one-step RT-PCR according to the instructions provided with the TaqMan Gold RT-PCR kit and the ABI7700 (Perkin-Elmer Applied Biosystems, Foster City, CA). For rRNA quantitation, the TaqMan Ribosomal RNA Control Reagent kit (P/N 4304483, Perkin-Elmer) was used according to the instructions provided, except that 600 nM of each primer and 100 nM of probe were used. Data analysis was done with Sequence Detector software (version 1.6.3, Perkin-Elmer Applied Biosystems) setting the baseline manually for each experiment and the threshold to 0.125 for CBL and 0.05 for rRNA. *Arabidopsis* RNA templates were treated with DNase I, RNase-free (Boehringer Mannheim, Indianapolis, IN).

CBL enzyme assays

Tissue for F_1 plants was frozen at −80 °C. For seedlings from soil, protein was extracted by grinding with a mortar and pestle in liquid nitrogen. For seedlings from plates, protein was extracted by grinding with a Caframo stirrer unit (RZR1, Heidolph, Kelheim, Germany), a blue polypropylene pellet pestle, and glass beads (Sigma, G9139) in microfuge tubes cooled in liquid nitrogen. Ground tissue was resuspended in 10 *m*M Tris pH 8.5, 10 μM pyridoxal 5-phosphate (PLP), spun for 10 min at 3800 × *g* to pellet debris. The supernatant was spun through a 0.45 μm filter (Millipore UFC30HV00), and concentrated with a 10 000 Da cutoff filter (Millipore, Microcon YM-10). The total concentration of protein in each sample was determined using a dye-binding method (BioRad Protein Assay) with bovine serum albumin as a standard. CBL activity was measured using a modified version of the method of Stijntjes *et al.* (1992). Extracted protein (6–8 μg in 5 μl) was combined with 1 μl of 30 mM L(+)cystathionine (Sigma) in 10 mM Tris pH 8.5, 10 μM PLP. Assays were performed in replicates of three to five in 1536 well plates (Greiner, black clear bottom). For each assay, control reactions with extracted protein but without substrate and with substrate but without extracted protein were done in parallel and the level of pyruvate detected was subtracted to compensate for endogenous pyruvate in the extracts and spontaneous decomposition of the substrate, respectively. Extracts from Col-0 plants were tested at multiple dilutions to create a standard curve. Plates were sealed (Beckman Biomek Seal and

Sample) and incubated for 3 h at 37 °C. The reaction was stopped with 3 μl 20 mM 4,5-dimethyl-*o*-phenylenediamine in 0.6 M HCl and derivatization of pyruvate by 4,5-dimethyl-*o*-phenylenediamine to form a quinoxalinol was allowed to proceed for 1 h at 37 °C. Fluorescence intensity was measured in a Tecan SpectraFluor with an excitation wavelength of 430 ± 10 nm and an emission wavelength of 535 ± 10 nm.

Luciferase assays

Leaf punches of uniform size from seedlings 3–4 weeks old or whole 2-week old seedlings, as indicated in the text, were ground in 0.1 M potassium phosphate, 1 mM DTT, pH 7.8 buffer and 20 μl of a 1:50 dilution of the extract were used to quantify the luciferase activity using the Promega Luciferase Assay Reagent (catalog number E1501). Relative light units (RLUs) were recorded with a Monolight 2010 luminometer (Becton Dickinson Microbiology System).

Photography and image processing

Plants were photographed with a DEI-750 video camera (Optronics Engineering, Goleta, CA) and images were captured with Scion Image (Scion Corporation, Frederick, MD) software. Images were adjusted for brightness, contrast, and color and assembled for figures with Adobe Photoshop (version 4.0) (San Jose, CA).

Results

Transgenic Arabidopsis *with cystathionine β-lyase constructs*

To directly compare the effect of inactivation of a gene by transgenic constructs with antisense, sense, or dsRNA, we placed a CBL gene fragment into a GAL4/C1 latent transactivation system (Guyer *et al.*, 1998) in each of these orientations. This system allows the generation of transgenic plants with the expression of a given sequence under the control of the GAL4 upstream activating sequence. In primary transformants, transcription of the given sequence should not occur and the plants will have a wild-type phenotype. In the case in which these plants are hemizygous for a single T-DNA insertion locus, these plants crossed with the GAL4/C1 effector line will produce F_1 progeny in which one half of the plants will express the given sequence. The other half of the F_1 progeny will have a wild-type phenotype because they do not inherit the transgenic construct with the upstream activating sequence controlling transgene expression. This strategy allows the identification of plants with potentially lethal phenotypes due to the inactivation of an essential gene. In the experiments described here, F_1 progeny were examined for altered phenotypes due to the changes in CBL gene expression.

Table 1. F_1 phenotypes for CBL constructs.

Construct	Affected/total	Phenotype
Antisense	0/23[a]	none
Sense	2/13	weak
dsRNA	10/11	weak to strong

Affected/total: number of lines affected divided by number of lines tested. Phenotypes are described in Results.
[a]Data generated with two different *Agrobacterium* strains (6 with AGL1 and 17 with GV3101).

Using the GAL4/C1 latent transactivation system, we compared the effects of inhibiting CBL expression with three constructs, shown in Figure 1, containing the same CBL gene fragment. In the dsRNA construct, CBL in the antisense orientation is proximal to the promoter followed by an 18 bp spacer and CBL in the sense orientation. This construct configuration was chosen to minimize any effects of expressing a portion of the CBL open reading frame; with nine stop codons and about 1200 bp before the CBL gene fragment in the sense orientation, translation of this message is likely to be highly reduced. In addition, the sense construct, which also contains the CBL start codon, serves as a control for the translation of this portion of CBL. The primary transformant plants generated from these three constructs were checked by PCR for the presence of a CBL gene fragment in the correct orientation (data not shown). The F_1 progeny from crosses of the primary transformants and the GAL4/C1 effector line were examined for altered phenotypes on MS + 2% sucrose plates. For the antisense construct, Table 1 shows that all of the F_1 progeny examined from 23 primary transformants (lines) had a wild-type phenotype. For the sense construct, F_1 progeny from two of 13 lines (lines 2-20 and 2-23) showed an abnormal phenotype (Table 1). On day 7 after placement in the growth room, 11 of 25 progeny from a single line (line 2-20) showed an abnormal phenotype. This phenotype consisted of decreased size, bleached and yellowed cotyledons, and increased purple coloration on margins of the cotyledons. On day 9, affected plants

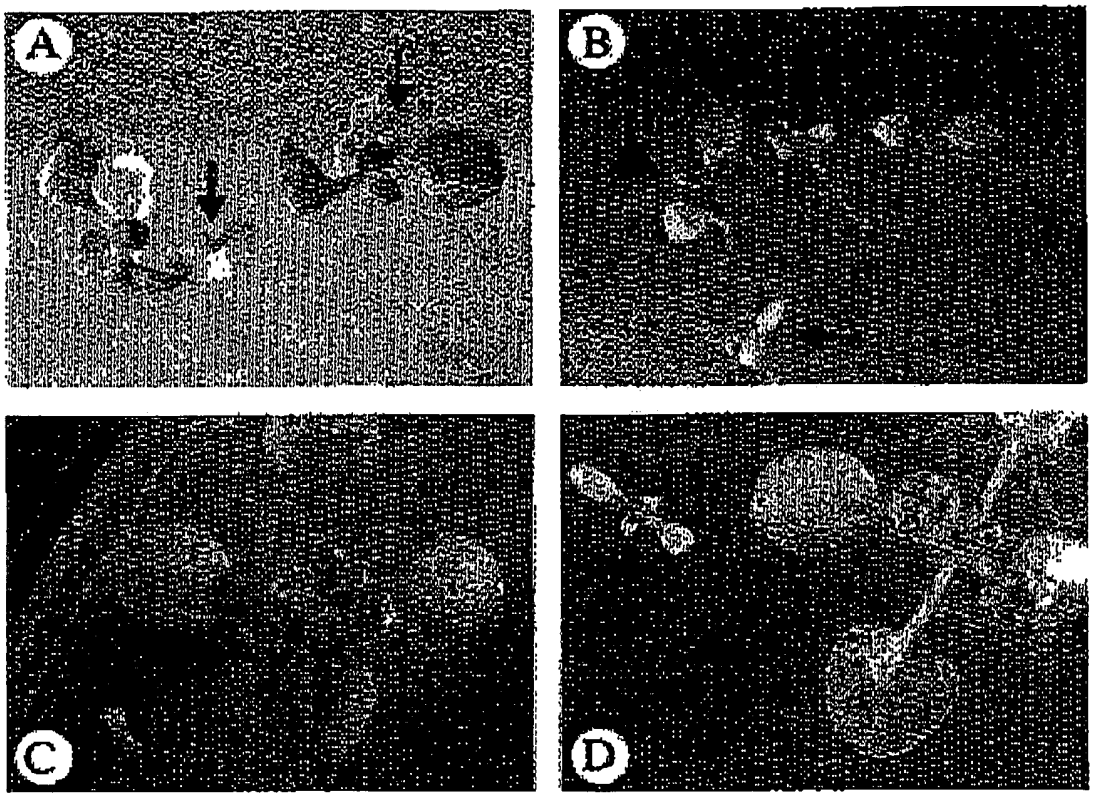

Figure 2. Phenotypes of F_1 progeny with decreased CBL expression. A. dsRNA line 3-12 on day 9. Two affected seedlings (arrows) are shown next to two normal siblings. B. dsRNA line 3-15 on day 9. Two seedlings are shown with a strong phenotype (arrows) and two with a weak phenotype (arrowheads). C. dsRNA line 3-13 on day 14. A seedling with strong, lethal phenotype (arrow) is shown next to a normal sibling. D. Sense line 2-20 on day 14. A seedling with weak phenotype (arrow) is shown next to a normal sibling. Photos are at the same magnification.

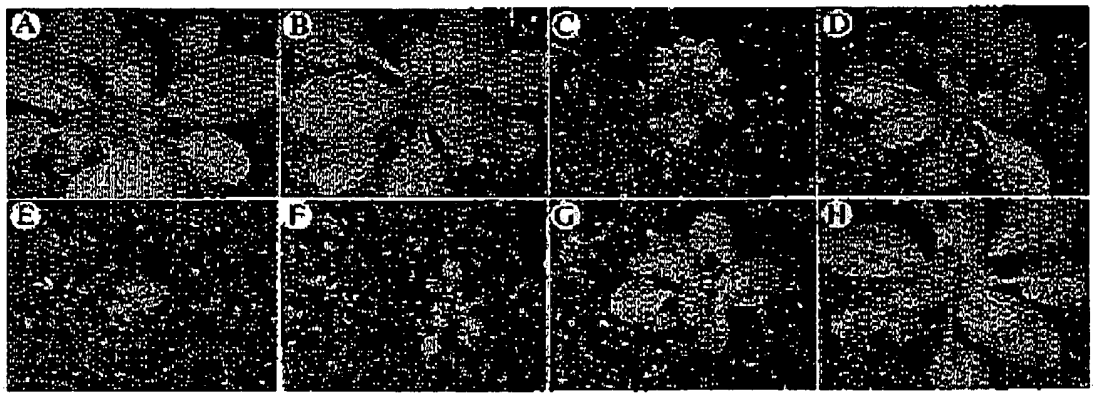

Figure 3. Phenotypes of F_1 progeny transplanted to soil. A. Antisense 1-4 plant. B. Sense 2-10 plant. C. Sense 2-20 plant. D. Sense 2-23 plant. E. dsRNA 3-15 plant. F. dsRNA 3-18 plant. G. dsRNA 3-21 plant. H. Col-0 control. All plants were grown for 15 days on plates and 17 days in soil under identical conditions. Plants in A, B, and H appear normal.

had no visible true leaves, while the wild-type plants had two true leaves. In addition, the length of the roots was reduced slightly. On day 14, Figure 2D shows the affected plants had only four or five small, pale true leaves compared to normal plants with five or six green true leaves. In addition, root growth along the bottom of the plate was reduced by about half in comparison with wild-type plants. Of the 27 F_1 progeny from sense line 2-23, 14 displayed a weaker, though similar, phenotype to that of progeny from line 2-20. On day 14, the affected 2-23 plants were smaller and slightly paler than wild type. Figure 3 shows the phenotypes of sense F_1 progeny 2-20C and 2-23C, which remained smaller than wild type, 17 days after transplantation to soil.

By contrast with the wild-type phenotype of the antisense lines and the weak, non-lethal phenotype of the sense lines, the dsRNA lines displayed abnormal phenotypes ranging from the weak phenotype of the sense lines to a strong lethal phenotype (Figure 2). For the dsRNA construct, F_1 progeny from 10 of 11 lines showed an abnormal phenotype (Table 1). On day 6, plants showed symptoms similar to those of line 2-20. On day 9, plants with a strong phenotype had decreased size, unexpanded pale cotyledons with increased purple coloration along their margins, no true leaves, and slightly decreased root growth (Figure 2A). Plants with weaker phenotypes showed small true leaves (Figure 2B). On day 11, the sickest progeny from line 3-15 showed bleached or brown small cotyledons and no true leaves. By day 14, it was clear that the seedlings with a strong phenotype were not viable as they had cotyledons with white and purple coloration and lacked true leaves (Figure 2C). As a control, four siblings of the dsRNA primary transformants without the dsRNA CBL transgene, as determined by PCR (data not shown) were also crossed to the GAL4/C1 effector line. All of the F_1 progeny from these crosses exhibited wild-type phenotypes (data not shown). Finally, the small, pale phenotype of some of the weaker F_1 progeny observed on plates remained similar after transplantation to soil (Figure 3).

Rescue of lethality by methionine

The abnormal phenotype observed in the CBL dsRNA lines was shown to be due to impaired methionine biosynthesis by the observation of essentially normal growth on methionine-containing media. We grew the F_1 progeny of the dsRNA CBL lines 3-15 and 3-18 on media supplemented with 150 μM or 250 μM L-methionine. All 12 seedlings on each methionine plate exhibited essentially normal growth in comparison with seedlings from the same lines grown side by side without methionine. Figure 4 shows representative plants from this experiment. All plants supplemented with methionine, including Col-0, showed a slightly slower rate of growth and decreased root length (data not shown). To verify that some of the dsRNA seedlings with a wild-type phenotype contained the CBL transgene, we transplanted all the seedlings from line 3-15 from plates with methionine to soil. After seven days, 10 of 12 of the seedlings developed a CBL dsRNA phenotype, i.e. yellow and brown leaves, decreased leaf growth, and smaller size, while transplanted Col-0 seedlings appeared normal (data not shown).

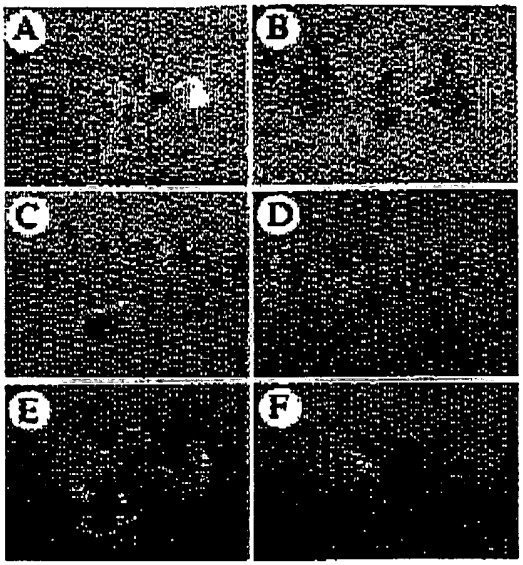

Figure 4. Rescue of CBL dsRNA phenotype by methionine. A and B. F_1 progeny of dsRNA line 3-18. C and D. F_1 progeny of dsRNA line 3-15. E and F. Col-0 seedlings. A, C, and E show seedlings grown without methionine. B, D, and F show seedlings grown with 150 μM methionine. Photos are at the same magnification. Seedlings are shown after 10 days of growth.

Quantitation of reduction of CBL mRNA and enzyme activity

We used RNA gel blots and TaqMan assays (Gibson *et al.*, 1996) to quantitate the levels of endogenous CBL mRNA in F_1 progeny containing the three

CBL constructs. The TaqMan assay is a 'real-time' quantitative RT-PCR method measuring PCR product accumulation with a dual-labeled fluorogenic probe. To quantitate the endogenous levels of CBL mRNA, we used probes that detected a portion of the 3' end of the cDNA that was not included in the transgenic constructs. In addition, we used a modified fluorescence assay (Stijntjes *et al.*, 1992) in a microtiter plate format to quantitate the levels of endogenous CBL activity in the extracts of F_1 progeny containing the three CBL constructs. The assay measured the amount of pyruvate formed after the β-cleavage of cystathionine by CBL. Based on the previous characterization of recombinantly produced *Arabidopsis* CBL (Ravanel *et al.*, 1996), we chose a substrate concentration (5 mM) that was significantly above the K_m (0.22 mM). In addition, we chose an incubation time for which the rate of product formation was linear with time for protein extracts prepared from Col-0 seedlings (data not shown). Thus, for wild-type and transgenic plants, the amount of activity measured under these conditions should have been proportional to the amount of active CBL.

Initially, we used RNA and protein isolated from F_1 plants grown on plates for about two weeks. We did not include plants with a wild-type phenotype in this analysis because there was not sufficient tissue for analysis of single plants at this stage and we wanted to avoid pooling plants with and without a CBL transgene. In addition, we also analyzed plants transplanted to soil, so that (1) we could directly compare plants with a wild-type phenotype to those with an abnormal phenotype and (2) we could check plants at a later stage of vegetative growth.

We observed reduced levels of CBL mRNA and activity in sense and dsRNA young seedlings compared with wild-type plants. Table 2 shows that the CBL mRNA levels were reduced to as low as 13% of wild-type levels in seedlings on plates. Using the fluorescence-based enzymatic CBL assay, we were able to reproducibly and accurately detect as little as 10% of wild-type activity (data not shown). We demonstrate that despite only 10% of wild-type CBL activity, F_1 plants from line 2-23 were viable with only a very weak phenotype. Lower levels of activity appeared to be deleterious to viability as lines 3-14, 3-15, and 3-22 all showed moderate to strong phenotypes and had activity levels below the detection limit of the enzymatic assay. In general, we saw a correlation between CBL mRNA and activity levels. Moreover, there was a correlation with phenotype and reduction

Table 2. Quantitation of CBL mRNA and enzyme levels in F_1 progeny on plates.

Line[a]	Phenotype	mRNA (TaqMan)[b], % of Col-0	Activity, % of Col-0[c]
2-20	weak	26	16
2-23	very weak	20	10
3-14	strong	15	<10
3-15	moderate	13	<10
3-20	strong	16	ND
3-21	moderate	18	ND
3-22	moderate	23	<10

Sense numbers begin with 2 and dsRNA line numbers begin with 3. Phenotype: 'very weak', 'weak', and 'strong' are described in Results; 'moderate' lines had a range from 'weak' to 'strong'. [a]RNA was isolated from affected F_1 progeny, with the healthiest 5 or 6 seedlings for each line not included, after 15 days in a growth room. In a separate experiment, protein was isolated from all affected seedlings for each line, after 13 days in a growth room.

[b]mRNA TaqMan vales were normalized with rRNA levels. CBL and rRNA levels were each measured in two independent experiments. In each CBL experiment, values were measured for 3 replicates at 3 RNA concentrations (9 replicates per experiment). In each rRNA experiment, values were measured for 3 replicates at 2 RNA concentrations (6 replicates per experiment).

[c]CBL mRNA and activity levels are reported as a percentage of that for Col-0 plants grown at the same time.

ND, not done.

in CBL mRNA and activity levels – generally, dsRNA lines had a greater reduction than sense lines in both CBL mRNA and activity levels (Table 2). The reported CBL mRNA and activity levels are averages of RNA and protein, respectively, isolated from several seedlings. In addition, the healthier seedlings were larger and contributed proportionately more mRNA and protein. As a result, the healthier seedlings, which have higher levels of CBL, make a greater contribution to the measured average CBL levels than the more affected seedlings. Thus, the CBL mRNA and activity levels in the most severely affected seedlings were probably less than the reported levels.

Although older seedlings also showed reductions in CBL mRNA and activity levels, there was less correlation between these reductions and plant phenotypes than was observed for the younger plants. For those lines with affected F_1 progeny, the healthiest affected seedlings on each plate were transplanted to soil, so that their mRNA and activity levels at the time of transplant might be expected to have been higher than those measured for the seedlings on plates in Table 2. After transfer of seedlings to soil, CBL mRNA

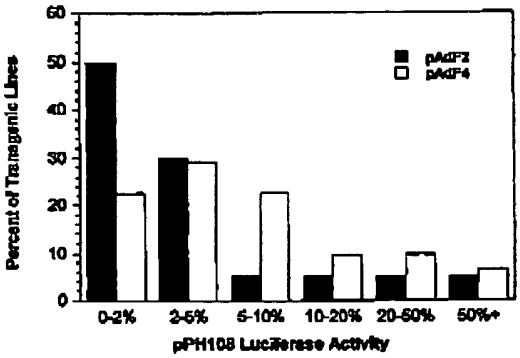

Figure 5. Distribution of luciferase activity levels for the pPH108 (pAdF2) and pPH108 (pAdF4) T_1 lines. Luciferase activity levels are shown as a percentage of the mean observed for 15 pPH108 plants. The levels measured in these 15 pPH108 plants had a mean of 182 000 RLU and ranged from 86 525 to 449 102 RLU. Both constructs can reduce luciferase levels as low as 1% of the pPH108 mean level. The pPH108, pPH108 (pAdF2), and pPH108 (pAdF4) plants are a mixture of hemizygotes and homozygotes for the pPH108 Luciferase-expressing transgene. Luciferase activity measured in RLU.

and activity levels may have changed due to other factors. We isolated RNA and protein from single plants grown in soil for 17 days after transplantation from plates. Phenotypes of representative plants are shown in Figure 3. Both TaqMan and RNA gel blot experiments showed that CBL mRNA levels are reduced (Table 3). The mRNA levels were not consistent with phenotype in every case (Figure 3). In particular, 1-3, 2-10, and 2-11 plants with wild-type phenotypes have lower CBL mRNA levels than 3-15 with a strong phenotype. Similar CBL mRNA levels were found by RNA gel blot when the mRNA levels of the β-tubulin, *TUB1*, gene were used for normalization rather than those of *ACT2* (data not shown). Similar to the mRNA results, CBL activity levels were reduced to as little as 15% of wild-type levels, but did not always correlate well with phenotype (Table 3 and Figure 3). Although the plants with strong phenotypes, such as 3-15 and 3-18, had 15 to 20% of wild-type CBL activity, plants with weak or very weak phenotypes, such as 2-20 and 2-23, also had similar activity levels. Some of the differences between mRNA and activity levels measured in Table 3 for a single line may be the result of analyzing one F_1 plant for mRNA level and a second F_1 plant for enzyme activity.

Luciferase activity in transgenic dsRNA Arabidopsis

In experiments parallel to those for CBL, we tested the effect of introducing constructs producing both the sense and antisense orientations of the luciferase gene in a single RNA transcript on a second highly expressed transgenic luciferase construct. Constructs pAdF2 and pAdF4 produce a dsRNA luciferase transcript under the control of the *ACT2* promoter (An *et al.*, 1996). pAdF4 has only 6 bp between the sense and antisense fragments, while pAdF2 contains the *bar* gene in between the two fragments (Figure 1). These constructs were introduced into *Arabidopsis* line pPH108, which expresses a high level of luciferase, to produce 20 pPH108 (pAdF2) and 31 pPH108 (pAdF4) double-transgenic independent T_1 seedlings. Figure 5 shows that a large proportion of these T_1 plants had a significant reduction in their luciferase activity. We measured the luciferase activity from leaf punches in all the T_1 double-transgenic seedlings, 15 independent pPH108 seedlings without an additional construct, and 32 T_1 Col-0 seedlings transformed with either pAdF2 or pAdF4. We observed a range of luciferase levels from 86 525 to 449 102 RLU for the 15 pPH108 seedlings (data not shown). Because the pPH108 plants used to initiate these experiments were not homozygous, it seems likely that there are variations in the luciferase levels due to copy number differences of the luciferase transgene between homozygotes and hemizygotes. The average luciferase level in the 15 pPH108 seedlings was used for comparison with the levels measured in the double transgenic lines. The luciferase levels for 16 transgenic Col-0 (pAdF2) and 16 Col-0 (pAdF4) lines did not exceed 0.2% of the pPH108 average (data not shown). Figure 5 shows that 80% of the pPH108 (pAdF2) T_1 seedlings had less than 5% of the average level of luciferase activity seen in the pPH108 line. Similarly, 52% of the pPH108 (pAdF4) T_1 plants retained less than 5% of the pPH108 luciferase activity. This strong silencing of the luciferase gene was found to be similar in the T_2 generation of 10 of 10 pPH108 (pAdF2) and 15 of 15 pPH108 (pAdF4) lines examined (data not shown).

Luciferase mRNA levels in transgenic dsRNA Arabidopsis

RNA gel blot analysis of plants transformed with the dsRNA constructs showed a majority of lines with very reduced luciferase mRNA levels and high dsRNA

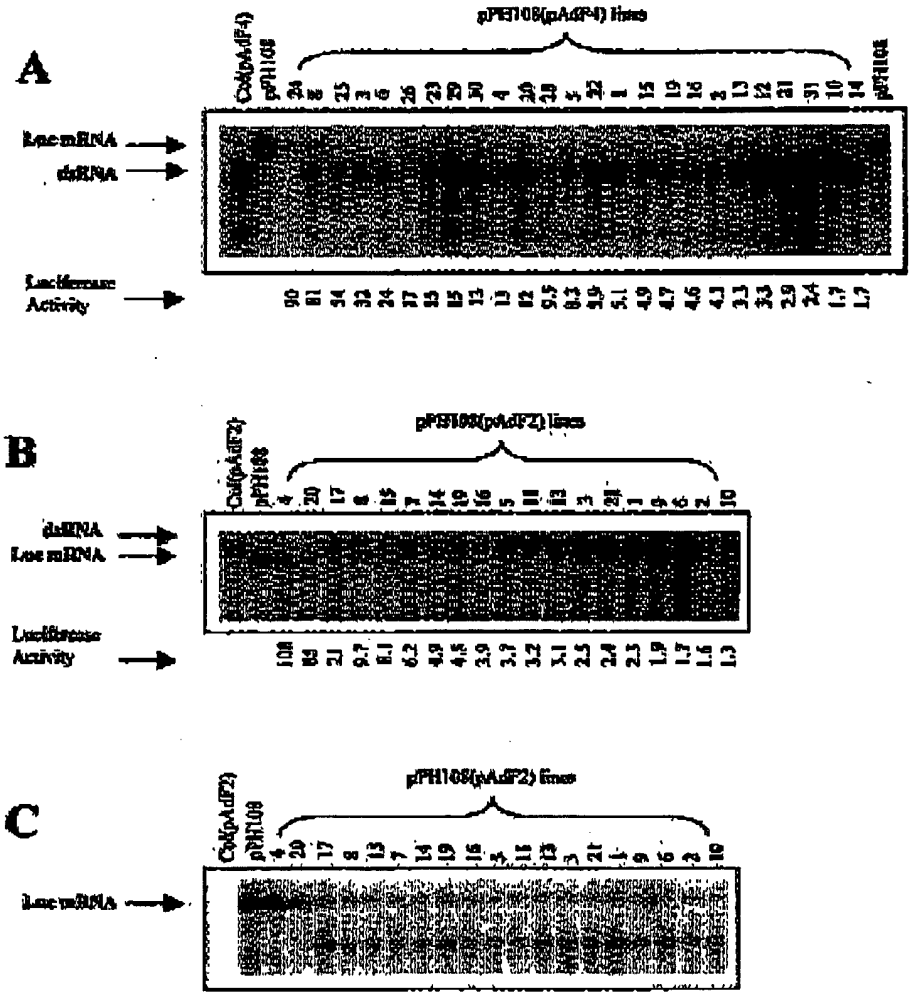

Figure 6. RNA gel blot analysis of pPH108 plants transformed with dsRNA constructs pAdF2 and pAdF4. Total RNA (4 μg per lane) was loaded and transferred to GeneScreen. Ethidium bromide staining of the agarose gels prior to blotting was done to confirm integrity of the RNA and loading of similar amounts of RNA (data not shown). A, pAdF4. The leftmost lane contains RNA from transgenic Col-0 pAdF4 and the second and rightmost lanes contain RNA from pPH108. B and C, pAdF2. The leftmost lane contains RNA from transgenic Col-0 pAdF2 and the second lane contains RNA from pPH108. Numbers above the other lanes indicate the line number. RNA in the other lanes was loaded in order of decreasing luciferase activity from left to right. Numbers below the other lanes indicate the measured luciferase activity (values shown are derived from actual values divided by 1000). In A and B, the probe consisted of the 3′-end portion of the luciferase gene present in both dsRNA constructs. In C, the probe contained the 5′ end of the luciferase gene, which was not included in the dsRNA constructs. Arrows indicate the luciferase and dsRNA transcripts. Transcripts produced by pAdF2 and pAdF4 are labeled as dsRNA, although their double-stranded nature has not been shown.

Table 3. Quantitation of CBL mRNA levels and enzyme levels in F_1 progeny transplanted to soil.

Line[a]	Phenotype	mRNA (TaqMan)[b], % of Col-0[c]	mRNA (RNA gel blot)[c], % of Col-0[c]	Activity[d], % of Col-0[c]
1-3	wild type	30	29	32
1-4	wild type	73	66	ND
1-6	wild type	46	41	ND
1-9	wild type	81	64	ND
1-20	wild type	35	28	ND
2-10	wild type	26	20	84
2-11	wild type	27	11	ND
2-12	wild type	58	39	ND
2-20	weak	15	8	19
2-23	very weak	14	9	18
3-15	strong	35	39	15
3-18	strong	19	ND	20
3-21	weak	25	16	41

Lines: 1, antisense; 2, sense; 3, dsRNA.
Phenotype: 'very weak', 'weak', and 'strong' are described in Results. 'Moderate' lines ranged between 'weak' and 'strong'.
[a]For a given line, RNA was isolated from a single plant and protein isolated from a second plant after growing for 15 days on plates and 17 days in soil.
[b]mRNA TaqMan values were normalized with rRNA levels. For CBL, values were measured for 3 replicates at 3 RNA concentration (9 replicates). For rRNA, values were measured for 3 replicates at 2 RNA concentrations (6 replicates).
[c]RNA gel blot mRNA levels were measured from PhosphorImager 445SI (Molecular Dynamics, Sunnyvale, CA) images and quantitated with Lab Gel software (Signal Analytics, Vienna, VA). CBL northern values were normalized with ACT2 mRNA levels. Values listed are the average of 4 blots.
[d]CBL activity levels are the average of two experiments.
[e]CBL mRNA and activity levels are reported as a percent of that for Col-0 plants grown at the same time.
ND, not done.

transcript levels. Total RNA isolated from the double-transgenic lines, Columbia lines with either dsRNA construct, and pPH108 seedlings was analyzed by RNA gel blot using a probe consisting of the portion of the luciferase gene present in pAdF2 and pAdF4. As shown in Figures 6A and 6B, the dsRNA transcript is detectable in 17 of 19 pPH108 (pAdF2) and 24 of 25 of pPH108 (pAdF4) double-transgenic lines. Plants without detectable dsRNA transcripts also showed the highest level of luciferase activity. Figure 6B reveals an inverse correlation between the level of dsRNA transcript and the level of the reporter luciferase mRNA. The latter also correlates with the level of luciferase activity. This relationship is less clear in Figure 6A in which several lines (26, 29, 30, 20, 22 and 10) depart from these trends. Because the probe used in the blots shown in Figures 6A and 6B hybridized to both the luciferase and the dsRNA transcripts, it was difficult to assess accurately the luciferase mRNA levels. When the blot shown in Figure 6B was stripped and re-hybridized with a luciferase probe homologous to a 5′ region of the gene not present in the dsRNA constructs, clear reductions in the levels of luciferase mRNA were observed (Figure 6C). T_1 plants with low levels of luciferase activity (less than 10% of the pPH108 control) had the lowest levels of luciferase mRNA (Figures 6B and 6C). It is noteworthy that the mobility of both the RNAs labeled 'dsRNA' in Figures 6A and 6B corresponds to the size expected. This result suggests that in the most abundant RNA species detected the *bar* gene spacer is not processed from the dsRNA transcripts and that transcription proceeds efficiently through the constructs. In addition, there does not appear to be any variation in the size of these dsRNA transcripts among the different lines, indicating that these constructs seem to be stably maintained.

Testing for inactivation of the bar *gene in transgenic* Arabidopsis *with a dsRNA luciferase construct*

Is any sequence in a dsRNA transcript able to induce silencing of its homologues or is this silencing limited to those sequences present in both sense and antisense copies? We were able to address this question with the pAdF2 construct, which displayed effective silencing of a second luciferase transgene and contains a *bar* gene between sense and antisense luciferase fragments. To determine whether this *bar* gene could inactivate a second, unlinked *bar* transgene, we designed an experiment in which pPH108 (pAdF2) plants were crossed with plants containing an active *bar* transgene. To obtain a suitable parent for the cross, we first grew T_2 seeds for all 20 pPH108 (pAdF2) lines on plates containing Basta to determine whether the *bar* gene was functional in these plants. All the seedlings showed Basta sensitivity, indicating that the *bar* transgene is not functional despite expression of RNA containing the *bar* open reading frame (Figure 6B). Second, we grew hygromycin-resistant T_2 plants for line 9 pAdF2 (pPH108), which had strong inactivation of the luciferase gene (Figure 6B). Two selected plants were homozygous for the dsRNA luciferase construct and hemizygous for the original luciferase transgene based on their segregation of hygromycin and kanamycin-resistant progeny, respectively (data not shown). These two pAdF2 plants were crossed with Basta-resistant T_3 plants from lines 17039 and 17183, which were homozygous for the *bar* gene-containing CSA104 T-DNA (D. Patton, personal communication). Both CSA104 lines contain a single genetic locus encoding Basta resistance. Table 4 shows that in the F_1 progeny of three different crosses, no Basta-sensitive progeny were observed; thus, there was no indication of inactivation of the CSA104 active *bar* transgene by the pAdF2 inactive *bar* gene. By PCR, we verified that the *bar* gene from pAdF2 was present in these F_1 progeny (data not shown). Luciferase levels in these F_1 plants were at either background levels or low levels equivalent to line 9 (Table 4). The former indicates the luciferase-containing that pPH108 construct was not present in the plant and the latter indicates that pPH108 was present, but that the luciferase gene was silenced by the pAdF2 construct. Control experiments showed that crossing either pAdF2 or CSA104 plants to Col-0 wild-type plants did not alter the detectable *bar* gene activity in F_1 progeny (data not shown).

Discussion

In this paper, we demonstrate that transgenic dsRNA constructs expressing gene fragments in both the sense and antisense orientation are more consistent and effective in gene silencing than those expressing only one of the two (Table 1). Previously, it has been shown that expression of antisense and sense RNAs for a viral or marker gene is effective for gene silencing in plants (Waterhouse *et al.*, 1998). Our work extends these findings on the dsRNA method in plants in three ways. First, we show inactivation of CBL, an endogenous plant gene. Inactivation of an endogenous gene is often more difficult to achieve than inactivation of a non-endogenous gene, in part because alterations in the expression of metabolic pathway genes, such as CBL, are often subject to compensatory mechanisms in the plant (see below). In addition, inactivation of endogenous genes is a valuable tool for determining gene function. Second, we demonstrate that gene silencing occurs in the presence of a spacer sequence between the sense and antisense gene fragments (Figure 5). In fact, it appears that the pAdF2 construct with the larger spacer may silence luciferase better than the pAdF4 construct with a very short spacer (Figure 5). This finding suggests that constructs expressing more complex, nested sets of gene sequences in a single transcript might be used to examine silencing, for example, sense and antisense copies of more than one gene. Finally, we have examined the reduction of mRNA levels in multiple lines for each construct to show that mRNA levels are reduced in dsRNA-expressing plants to levels of about 10% in the case of CBL, but not below the level of detection for either CBL or luciferase. This observation shows some parallels with what has been found in *C. elegans* injected with dsRNA in which some nuclear mRNA remains, though cytoplasmic mRNA is not detected (Montgomery *et al.*, 1998).

Previous studies have raised the issue of whether gene silencing in plants with gene sequences present as inverted repeats acts through ectopic DNA pairing or a RNA mechanism (Bender and Fink, 1995; Baulcombe and English, 1996; Metzlaff *et al.*, 1999; Kooter *et al.*, 1999). Recent studies of *Petunia* chalcone synthase antisense transgenic lines suggest that lines with two T-DNAs arranged as inverted repeats may cause silencing by producing dsRNA transcripts (Stam *et al.*, 2000). Our results with the dsRNA CBL construct strongly suggest that gene silencing occurred through the action of a RNA containing both

Table 4. Basta resistance and luciferase phenotypes for pAdF2 F_1 progeny.

pAdF2 parent	Bar^R parent[a]	Bar^S F_1 / total F_1[b]	Luciferase 'silenced'/ total[c]
9A	17183-3A	0/22	6/22[d]
9B	17049-4C	0/20	13/20[e]
9A	17183-3B	0/29	ND

F_1 progeny from pAdF2/pAdF2 pPH108/- T_2 plants crossed with Bar^R/Bar^R T_3 plants were grown on germination medium containing 10 μg/ml Basta.
[a]Plants contain CSA104 with an active *bar* gene (see Results).
[b]Bar^S plants would indicate inactivation of the *bar* gene by pAdF2.
[c]Luciferase 'silenced' seedlings have low, non-background levels (>300 RLU) of luciferase. Plants with background levels (<300 RLU) are likely to lack the pPH108 construct.
[d]Luciferase assays were done on whole 2-week old seedlings.
[e]Luciferase assays were done on single leaves from 4-week old plants.
ND, not done.

the antisense and sense CBL fragments and not ectopic DNA pairing. By using a transactivation system (Guyer *et al.*, 1995), we have generated T_1 plants that should have inverted repeats, but no transcription of the CBL transgenic construct. Those T_1 plants are indistinguishable from wild-type plants (data not shown). By crossing those T_1 plants with GAL4/C1-expressing plants, we generated T_1 plants that contain both the dsRNA construct and GAL4/C1 and exhibit a visible phenotype (Figure 3). Thus, the phenotypic effects that we observe are dependent on the transcription of the dsRNA transgene and are most probably due to the action of a dsRNA or its derivatives.

Although a construct with a gene sequence present in both antisense and sense orientations in a dsRNA transcript inactivates a second copy of the gene at high efficiency, the *bar* gene that we placed in the spacer region between the sense and antisense luciferase gene fragments behaved differently. When pPH108 (pAdF2) transgenic plants, which contain a non-functional *bar* gene, were crossed to plants containing a functional *bar* gene, the pAdF2 *bar* gene did not induce any detectable silencing of the second *bar* gene in the F_1 progeny (Table 4). Because either sense or antisense transcripts have been shown in many cases to silence genes (reviewed in Bruening, 1998), this result may indicate that there is a reduction in the efficiency of gene silencing by the *bar* gene in a dsRNA transcript, rather than a complete absence of silencing. Another possibility is that the formation of a hairpin structure promotes the silencing of the gene with two copies, but reduces the silencing potential of the gene in the loop region. While this result was obtained with only one construct, we would like to consider its implications in the light of existing models for RNA-triggered gene silencing (Fire, 1999). In one class of model, a RNA-dependent RNA polymerase produces cRNA that hybridizes to target mRNA and leads to the degradation of the target mRNA by a ribonuclease (Jorgensen *et al.*, 1998; Waterhouse *et al.*, 1998). In this case, the cRNA molecules may not be transcribed as efficiently through the single-stranded portion of the transcript. Alternatively, the cRNA transcripts might be as short as 25 nucleotides (Hamilton and Baulcombe, 1999), and may be initiated exclusively in the double-stranded regions. In a second class of model, there is a dsRNA-degrading protein complex, but it does not involve any copying of the dsRNA. Recent results with a *Drosophila* cell-free system show that sequence-specific mRNA degradation can be triggered by the addition of dsRNA, which is cleaved efficiently to 21 to 23 nucleotide fragments that may be the active agents in initiating mRNA degradation (Tuschl *et al.*, 1999; Zamore *et al.*, 2000). Replacement of the dsRNA with single-stranded RNAs (ssRNAs) in this system does not lead to efficient mRNA degradation (Tuschl *et al.*, 1999). Such a model would suggest that silencing is not triggered efficiently in our experiment with the single-stranded portion of the transcript because no active agents are generated. It is possible that the ssRNA *bar* portion of the transcript is not cleaved efficiently into the active small RNA fragments or that these RNA fragments are not stable. Alternatively, dsRNA-specific proteins may not be attached properly to the single-stranded *bar* RNA fragments preventing them from triggering mRNA inactivation. Further experiments will be necessary to determine under what circumstance, if any, sequences in a spacer region adjacent to an inverted repeat can be silenced.

We demonstrate that a construct expressing a sense and an antisense copy of a gene appears to be stably transmitted in *Arabidopsis*. Such a property is important if this type of method is to be used for the development of transgenic crops. For the luciferase constructs, we showed that both the T_1 and T_2 plants exhibited reduced levels of luciferase. In addition, the F_1 progeny of T_2 plants were shown to contain the *bar* gene from the pAdF2 construct by PCR (data not shown) and exhibited reduced luciferase levels (Table 4). For the CBL construct, we showed that an intact dsRNA construct was detected by PCR in the T_1

generation and was more active in the F_1 progeny of these T_1 plants compared to plants with only an antisense or sense construct. Moreover, dsRNA-mediated silencing of luciferase activity was eliminated in the T_2 progeny that did not inherit the pAdF2 construct (data not shown). Thus, silencing does not seem to be inherited from parents with dsRNA silencing if the progeny do not contain the dsRNA construct.

Methionine biosynthesis

We demonstrate in this study that the CBL gene is essential for growth of *Arabidopsis*. In addition, we show that this essentiality is due to its role in methionine biosynthesis because plants with reduced CBL levels can be rescued by supplying exogenous methionine. Although there is a report of CBL activity loss in a *Nicotiana plumbaginifoli* mutant (Negrutiu *et al.*, 1985), the underlying defect was not shown to be in the gene encoding this enzyme. In addition, L-aminoethoxyvinylglycine (AVG) has been reported to be an inhibitor of CBL with herbicidal activity in *Lemna* and this activity is partially reversed by exogenous methionine (Datko and Mudd, 1982). While this suggested that CBL is essential for *Lemna*, AVG has been reported to inhibit other enzymes as well (Ravanel *et al.*, 1998), so that the lethality associated with AVG treatment may be caused by inhibition of other essential enzymes in the plant. We also show that *Arabidopsis* plants with CBL activity levels reduced to about 10% do not grow normally, but remain viable. This result is comparable to that found for CGS, the enzyme immediately prior to CBL. Chemical inhibition of CGS by propargylglycine (PAG) showed that *Lemna* plants grew normally with CGS activity at or above 16% of control activity and were inviable with CGS activity below 10% (Thompson *et al.*, 1982).

Our observation that CBL enzyme activity levels do not always correlate with the strength of the phenotype of the transgenic plants suggests that there could be other mechanisms acting to maintain the health of the plant during methionine starvation. It is possible that an increase in the level or activity of other enzymes involved in methionine biosynthesis could be compensating for lowered levels of CBL. Evidence for such compensation during methionine starvation has been demonstrated for *Lemna* plants able to tolerate increased levels of PAG, an inhibitor of CGS, after growth in sub-lethal doses of PAG (Thompson *et al.*, 1982). In addition, *Lemna* plants treated with 0.05 μM AVG showed a two-fold increase in CGS specific activity (Thompson *et al.*, 1981). Because the stability of CGS mRNA plays a key role in regulating methionine levels in *Arabidopsis* (Chiba *et al.*, 1999), it seems possible that plants with decreased levels of CBL may have increased levels of CGS mRNA. Further, *Arabidopsis* hemizygous for a CGS antisense construct were reported to have a three-fold increase in CBL (Ravanel *et al.*, 1998). Regulation of genes outside of the methionine biosynthetic pathway in response to methionine starvation or other changes in metabolite levels is another possible strategy plants may employ to compensate for the decreased levels of CBL. This possibility is supported by findings that chemical inhibition of the histidine biosynthetic pathway results in altered mRNA levels for genes involved in the biosynthesis of other amino acids (Guyer *et al.*, 1995). Finally, CBL levels may increase over time as multiple compensatory mechanisms are induced. In plants with no visible phenotype, but low levels of CBL (Tables 2 and 3), there may be two factors responsible. First, there may not be any starvation for methionine initially and the plant may be able to build up sufficient methionine to prevent any visible phenotype in older plants. Because older plants have more cell expansion and less cell division, they might have lower methionine requirements. Second, the absence of starvation for methionine may prevent the activation of any compensatory mechanisms to increase CBL levels. It remains unresolved why such compensation mechanisms might have varied among the plants analyzed in this study.

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In the final stages of preparation of this manuscript, similar findings with constructs designed to produce double-stranded RNA were reported (Chuang and Meyerowitz, 2000).

References

An, Y.Q., McDowell, J.M., Huang, S., McKinney, E.C., Chambliss, S. and Meagher, R.B. 1996. Strong, constitutive expression of the *Arabidopsis ACT2/ACT8* actin subclass in vegetative tissues. Plant J. 10: 107–121.

Baulcombe, D.C. and English, J.J. 1996. Ectopic pairing of homologous DNA and post-transcriptional gene silencing in transgenic plants. Curr. Opin. Biotechnol. 7: 173–180.

Bechtold, N., Ellis, J. and Pelletier, G. 1993. *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris 316: 1194–1199.

Becker, D., Kemper, E., Schell, J. and Masterson, R. 1992. New plant binary vectors with selectable markers located proximal to the left T-DNA border. Plant Mol. Biol. 20: 1195–1197.

Bender, J. and Fink, G.R. 1995. Epigenetic control of an endogenous gene family is revealed by a novel blue fluorescent mutant of *Arabidopsis*. Cell 83: 725–734.

Bork, C. and Hell, R. 1997. Cloning and expression of the CBL1 gene encoding cystathionine-β-lyase from *Arabidopsis thaliana*. Plant Physiol. 115: 864.

Bruening, G. 1998. Plant gene silencing regularized. Proc. Natl. Acad. Sci. USA 95: 13349–13351.

Chiba, Y., Ishikawa, M., Kijima, F., Tyson, R.H., Kim, J., Yamamoto, A., Nambara, E., Leustek, T., Wallsgrove, R.M. and Naito, S. 1999. Evidence for autoregulation of cystathionine γ-synthase mRNA stability in *Arabidopsis*. Science 286: 1371–1374.

Chuang, C.F. and Meyerowitz, E.M. 2000. Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 97: 4985–4990.

Cogoni, C. and Macino, G. 1999. Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature 399: 166–169.

Datko, A.H. and Mudd, S.H. 1982. Methionine biosynthesis in *Lemna*: inhibitor studies. Plant Physiol. 69: 1070–1076.

D'Halluin, K., De Block, M., Denecke, J., Janssens, J., Leemans, J., Reynaerts, A. and Botterman, J. 1992. The *bar* gene as selectable and screenable marker in plant engineering. Meth. Enzymol. 216: 415–426.

Fire, A. 1999. RNA-triggered gene silencing. Trends Genet. 15: 358–363.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391: 806–811.

Gibson, U.E.M., Heid, C.A. and Williams, P.M. 1996. A novel method for real time quantitative RT-PCR. Genome Res. 6: 995–1001.

Grant, S.R. 1999. Dissecting the mechanisms of posttranscriptional gene silencing: divide and conquer. Cell 96: 303–306.

Guyer, D., Patton, D. and Ward, E. 1995. Evidence for cross-pathway regulation of metabolic gene expression in plants. Proc. Natl. Acad. Sci. USA 92: 4997–5000.

Guyer, D., Tuttle, A., Rouse, S., Volrath, S., Johnson, M., Potter, S., Gorlach, J., Goff, S., Crossland, L. and Ward, E. 1998. Activation of latent transgenes in *Arabidopsis* using a hybrid transcription factor. Genetics 149: 633–639.

Hamilton, A.J. and Baulcombe, D.C. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286: 950–952.

Hamilton, A.J., Brown, S., Yumhai, H., Ishizuka, M., Lowe, A., Solis, A.-G.A. and Grierson, D. 1998. A transgene with repeated DNA causes high frequency, post-transcriptional suppression of ACC-oxidase gene expression in tomato. Plant J. 15: 737–746.

Jorgensen, R.A., Atkinson, R.G., Forster, R.L.S. and Lucas, W.J. 1998. An RNA-based information superhighway in plants. Science 279: 1486–1487.

Koncz, C. and Schell, J. 1986. The promotor of the TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet. 204: 383–396.

Kooter, J.M., Matzke, M.A. and Meyer, P. 1999. Listening to the silent genes: transgene silencing, gene regulation and pathogen control. Trends Plant Sci. 4: 340–347.

Lagrimini, L.M., Burkhart, W., Moyer, M. and Rothstein, S. 1987. Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: molecular analysis and tissue-specific expression. Proc. Natl. Acad. Sci USA 84: 7542–7546.

Lazo, G.R., Stein, P.A. and Ludwig, R.A. 1991. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. Bio/technology 9: 963–967.

Merlo, A.O., Cowen, N., Delate, T., Edington, B., Folkertz, O., Hopkins, N., Lemeiux, C., Skokut, T., Smith, K., Woosley, A., Yang, Y., Young, S. and Zwick, M. 1998. Ribozymes targeted to stearoyl-ACP Δ9 desaturase mRNA produce heritable increases of stearic acid in transgenic maize leaves. Plant Cell 10: 1603–1622.

Meins, M.F., van der Winden, J., Matzke, M.A. and Matzke, A.J.M. 1999. Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters *in trans*. EMBO J. 18: 241–248.

Minet, M., Dufour, M.E. and Lacroute, F. 1992. Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. Plant J. 2: 417–422.

Montgomery, M.K., SiQun, X. and Fire, A. 1998. RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA 95: 15502–15507.

Negrutiu, I., De Brouwer, D., Dirks, R. and Jacobs, M. 1985. Amino acid auxotrophs from protoplast cultures of *Nicotiana plumbaginifolia*, Viviani. Mol. Gen. Genet. 199: 330–337.

Newmann, T., de Bruijn, F.J., Green, P., Keegstra, K., Kende, H., McIntosh, L., Ohlrogge, J., Raikhel, N., Somerville, S., Thomashow, M., Retzel, E. and Somerville, C. 1994. Genes galore: a summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. Plant Physiol. 106: 1241–1255.

Norris, S.R., Meyer, S.E. and Callis, J. 1993. The intron of *Arabidopsis thaliana* polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression. Plant Mol. Biol. 21: 895–906.

Ravanel, S., Gakiere, B., Job, D. and Douce, R. 1998. The specific features of methionine biosynthesis and metabolism in plants. Proc. Natl. Acad. Sci. USA 95: 7805–7812.

Ravanel, S., Job, D. and Douce, R. 1996. Purification and properties of cystathionine β-lyase from *Arabidopsis thaliana* overexpressed in *Escherichia coli*. Biochem. J. 320: 383–392.

Ravanel, S., Ruffet, M.L. and Douce, R. 1995. Cloning of an *Arabidopsis thaliana* cDNA encoding cystathionine *ß*-lyase by functional complementation in *Escherichia coli*. Plant Mol. Biol. 29: 875–882.

Schiebel, W., Pelissier, T., Riedel, L., Thalmeir, S., Schiebel, R., Kempe, D., Lottspeich, F., Sanger, H.L. and Wassenegger, M. 1998. Isolation of RNA-dependent RNA polymerase-specific cDNA clone from tomato. Plant Cell 10: 2087–2101.

Stam, M., de Bruin, R., van Blokland, R., van der Hoorn, R.A.L., Mol, J.N.M. and Kooter, J.M. 2000. Distinct features of post-transcriptional gene silencing by antisense transgenes in single copy and inverted T-DNA repeat loci. Plant J. 21: 27–42.

Stijntjes, G.J., te Koppele, J.M. and Vermeulen, N.P. 1992. High-performance liquid chromatography-fluorescence assay of pyruvic acid to determine cysteine conjugate beta-lyase activity: application to S-1,2-dichlorovinyl-L-cysteine and S-2-benzothiazolyl-L-cysteine. Anal. Biochem. 206: 334–343.

Thompson, G.A., Datko, A.H. and Mudd, S.H. 1982. Methionine synthesis in *Lemna*: Inhibition of cystathionine *γ*-synthase by propargylglycine. Plant Physiol. 70: 1347–1352.

Thompson, G.A., Datko, A.H., Mudd, S.H. and Giovanelli, J. 1981. Methionine biosynthesis in *Lemna*: studies on the regulation of cystathionine *γ*-synthase, *O*-phosphohomoserine sulfhydrylase, and *O*-acetylserine sulfhydrylase. Plant Physiol. 69: 1077–1083.

Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P. and Sharp, P.A. 1999. Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev. 13: 3191–3197.

Vaucheret, H., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.-B., Mourrain, P., Palauqui, J.-C. and Vernhettes, S. 1998. Transgene-induced gene silencing in plants. Plant J. 16: 651–659.

Verwoerd, T.C., Dekker, B.M. and Hoekema, A. 1989. A small-scale procedure for the rapid isolation of plant RNAs. Nucl. Acids Res. 17: 2362.

Wassenegger, M. and Pelissier, T. 1998. A model for RNA-mediated gene silencing in plants. Plant Mol. Biol. 37: 349–362.

Waterhouse, P.M., Graham, M.W. and Wang, M.-B. 1998. Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Natl. Acad. Sci. USA 95: 13959–13964.

Waterhouse, P.M., Smith, N.A. and Wang, M.-B. 1999. Virus resistance and gene silencing: killing the messenger. Trends Plant Sci. 4: 452–457.

Zamore, P.D., Tuschl, T., Sharp, P.A. and Bartel, D.P. 2000. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21-23 nucleotide intervals. Cell 101: 25–33.

Zhu, T., Peterson, D.J., Tagliani, L., St Clair, G., Baszczynski, C.L. and Bowen, B. 1999. Targeted manipulation of maize genes in vivo using chimeric RNA/DNA oligonucleotides. Proc. Natl. Acad. Sci. USA 96: 8768–8773.

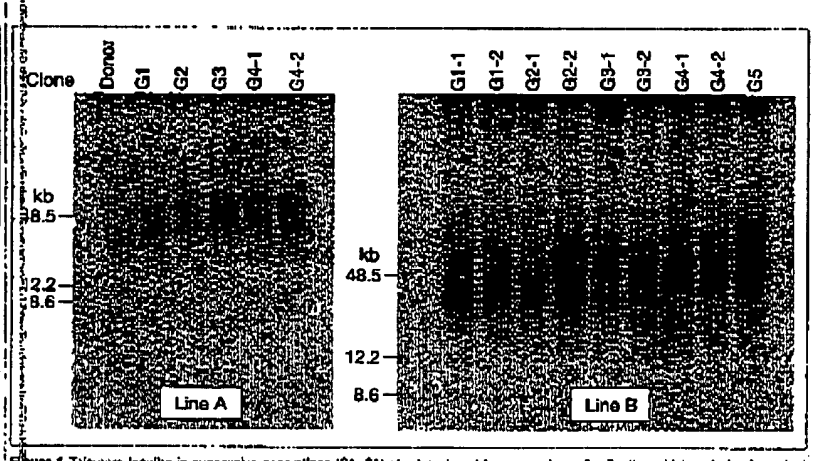

Figure 1 Telomere lengths in successive generations (G1–G5) of mice cloned from cumulus cells. Southern-blot analysis of terminal restriction-enzyme-cut fragments in five sequential generations shows that telomeres do not undergo incremental erosion in successive clonal generations. Genomic DNA isolated from peripheral-blood lymphocytes taken from representative animals from each generation was digested with the restriction enzyme *Hinf*I, resolved on a pulse field gel, transferred to a solid support and probed with a 5′-^{32}P-labelled $(T_2AG_3)_3$ oligonucleotide. Peripheral blood lymphocytes were sampled on the same day. Ages of mice (in months) were: in line A, donor, 18; G1, 16; G2, 14; G3, 12; G4, 9; G5, 9; in line B, G1, 15.5; G2, 13; G3, 11; G4, 9; G5, 7. Suffix numbers (G4-1 and G4-2, for example) identify different pups of each generation.

was repeated with cumulus cells from adult G1 mice as nucleus donors to produce the next clonal generation, G2, and so on. Table 1 summarizes the results obtained following the reconstruction of 3,920 enucleated oocytes.

Previously, about 2% of enucleated oocytes receiving a cumulus cell nucleus developed to live-born pups[1]. This value is comparable to the cloning efficiency for G1 in lines A (1.5%) and B (4.2%). However, the success rate dropped in successive cloned generations: line A did not produce a G5 clone from 670 reconstructed oocytes; in line B, the only live-born G6 clone was cannibalized by her foster mother, thereby terminating the line. Mouse lines A and B therefore represent totals of 9 and 26 clones from their respective donors. Placental size was consistently in the range previously reported for cloned mice[2] and did not increase in successive generations (data not shown).

Do sequentially cloned mice show signs of accelerated ageing? We assessed the behaviour of these mice and determined telomere lengths to assess organismal and cellular measures of ageing, respectively. We evaluated learning ability in the Morris water maze and Krushinsky tests, as well as strength and agility, and also used other assays designed to monitor signs of premature ageing, such as a decline in activity in the home cage and loss of coordination[4]. All cloned mice were, by these criteria, normal compared with age-matched controls (data not shown); the G5 mouse is alive and healthy at 1.5 years.

We measured telomere length in peripheral blood lymphocytes of clones G1–G6 by Southern-blot analysis of terminal restriction-enzyme-digested fragments (Fig. 1) and found no evidence of shortened telomeres in the cloned mice. In fact, our results show that the telomeres lengthen with each generation. As representative animals of each generation were sampled simultaneously, we cannot rule out an age-related contribution to this increase (with younger mice having longer telomeres). In addition, long telomeres in mice are optimally studied by means of different assays such as quantitative fluorescence *in situ* hybridization[5]. We have detected telomerase activity in cumulus cells (data not shown); it is therefore possible that telomeres in these cells are unusually long, resulting in offspring with concomitantly longer telomeres.

Shortened[6] and lengthened[7] telomeres have been reported in cloned livestock but, unlike ours, those experiments involved only a single round of cloning. Our results on sequentially cloned mice verify that telomere shortening is not a necessary outcome of the cloning process[8]. However, as only 1–2% of reconstructed oocytes yield live-born clones, the possibility of selection for donor nuclei with the longest telomeres cannot be excluded. Further investigation is required into the consequences of nuclear transfer on telomere length and lifespan.

Table 1 Effect of sequential cloning on full-term development

Line	G1	G2	G3	G4	G5	G6	Total
A	2/131 (1.5)	1/228 (0.4)	1/263 (0.4)	5/299 (2.1)	0/670 (0)	-	9/1,530 (0.6)
B	4/96 (4.2)	7/351 (2.0)	5/352 (1.4)	6/286 (2.1)	3/581 (0.5)	1/724 (0.1)	26/2,390 (1.1)

Successive generations are represented as G1, G2 and so on for two independent mouse lines, A and B. The number of pups born live after cumulus-cell nuclear transfer is compared to successfully reconstructed oocytes (pups/oocytes), with the corresponding percentages in parentheses. Significant χ^2 comparisons were derived for G4 and G5 from line A, G1 and G5, G6 from line B, and G2, G3, G4 versus G5 from line B ($P < 0.05$).

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1. Wakayama, T., Perry, A. C. F., Zuccotti, M., Johnson, K. R. & Yanagimachi, R. *Nature* 394, 369–374 (1998).
2. Wakayama, T. & Yanagimachi, R. *Nature Genet.* 22, 127–128 (1999).
3. Wakayama, T., Rodriguez, I., Perry, A. C. F., Yanagimachi, R. & Mombaerts, P. *Proc. Natl Acad. Sci. USA* 96, 14984–14989 (1999).
4. Tamashiro, K. L., Wakayama, T., Blanchard, R. J., Blanchard, D. C. & Yanagimachi, R. *Biol. Reprod.* 63, 328–334 (2000).
5. Zijlmans, J. M. *et al. Proc. Natl Acad. Sci. USA* 94, 7422–7428 (1997).
6. Shiels, P. G. *et al. Nature* 399, 316–317 (1999).
7. Lanza, R. P. *et al. Science* 288, 665–669 (2000).
8. Wilmut, I., Clark, J. & Harley, C. B. *Nature Biotech.* 18, 599–600 (2000).

Gene expression

Total silencing by intron-spliced hairpin RNAs

Post-transcriptional gene silencing (PTGS), a sequence-specific RNA degradation mechanism inherent in many life-forms, can be induced in plants by transforming them with either antisense[1] or co-suppression[2] constructs, but typically this results in only a small proportion of silenced individuals. Here we show that gene constructs encoding intron-spliced RNA with a hairpin structure can induce PTGS with almost 100% efficiency when directed against endogenous

brief communications

genes. These constructs could prove valuable in reverse genetics, genomics, engineering of metabolic pathways and protection against pathogens.

Induction of PTGS by co-suppression and antisense methods that target the Nia-protease (*Pro*) gene sequence of potato virus Y (PVY)[3] cause silencing in 7% and 4% of independent transformants, respectively; induction of PTGS in these tobacco plants (*Nicotiana tabacum*) manifests as immunity[1,4] to the virus.

Using principles we developed for silencing constructs that express double-stranded RNA and inverted-repeat RNAs[5], we made a construct encoding a single self-complementary hairpin RNA (hpRNA) of the *Pro* sequence. The construct contains sense and antisense *Pro* sequences flanking an 800-nucleotide spacer fragment derived from the *uidaA* (GUS) gene (Fig. 1a). About 60% (25/43) of the plants that are transformed with this construct, many of which contained a single transgene copy, were immune to the virus. The spacer fragment contributed to the stability of the perfect inverted-repeat sequences, but it was not required for the specificity of the PTGS (Fig. 1a).

To test the effect of removing the loop region of hpRNA, we replaced the spacer with an intron sequence (Fig. 1a, b). The intron sequence provides stability to the DNA, but is spliced out during pre-mRNA processing[5] to produce loopless hpRNA. As a control, we made a sister construct in which the intron sequence was inserted in the reverse, non-splicing, orientation. When transformed into tobacco, 22 of 34 (65%) reverse-intron plants were immune, a similar frequency to plants transformed with the GUS spacer construct. Amazingly, we found that 22 of 23 plants transformed with the construct containing the functional intron were immune to the virus.

To test whether this enhancement by the sense-intron construct was a general phenomenon, we made two hpRNA constructs against the endogenous Δ12-desaturase (*fad2*) gene of *Arabidopsis*, which catalyses the conversion of oleic to linoleic acid in the seed[6,7]; one construct contained an intron and the other a non-intron spacer region. We found that 69% (44/63) of the transgenic plants with the non-intron spacer region construct showed PTGS of the Δ12-desaturase gene, but that 100% (30/30) of plants transformed with the intron construct showed silencing of this gene.

How does the presence of this intron enhance silencing efficiency? The process of intron excision from the construct by the spliceosome might help to align the complementary arms of the hairpin in an environment favouring RNA hybridization, promoting the formation of a duplex. Alternatively, splicing may transiently increase the amount of hairpin RNA by facilitating, or retarding, the hairpin's passage from the nucleus, or by creating a smaller, less nuclease-sensitive loop.

Our PVY constructs contained intron-2 from the *Pdk* gene of *Flaveria*[8], whereas the Δ12-desaturase construct contained intron-1 from the *Arabidopsis Fad2* gene (Fig. 1b). PVY constructs were controlled by the constitutive CaMV35S (ref. 9) promoter and produced hpRNA containing the PVY coding-region sequence (700 nucleotides); the desaturase gene construct used the seed-specific napin promoter[10] to produce hpRNA representing 120 nucleotides of the 3′-untranslated region of the Δ12-desaturase gene.

We believe that constructs encoding intron-hpRNA should efficiently induce PTGS for a wide range of genes in a variety of circumstances and could become as useful to plant biology as RNAi[11,12] is to the study of nematodes and *Drosophila*. The transgene design might also have application in organisms in which RNAi has been obtained by injection of double-stranded RNA.

a Gene construct	Predicted structure of RNA transcript	% PTGS	*n*
Sense PVY*	→	7	27
Antisense PVY*	←	4	25
hpRNA (PVY arms, GUS loop)	← ⟲ →	58	43
hpRNA (PVY arms, reverse intron)	← ⟲ →	65	34
hpRNA (PVY arms, sense intron)	← ⟲ →	86	23
Sense Δ12-desaturase†	→	10	41
Antisense Δ12-desaturase†	←	15	21
hpRNA (Δ12 arms, spacer loop)	← ⟲ →	69	63
hpRNA (Δ12 arms, intron)	← ⟲ →	100	30

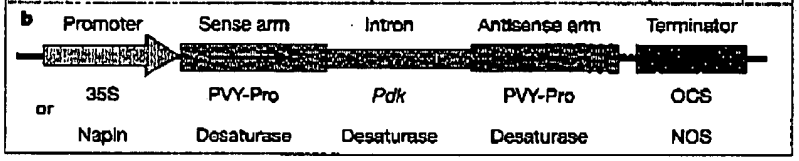

Figure 1 Efficiency of induction of post-transcriptional gene silencing (PTGS) by different gene constructs and the predicted structure of RNA transcribed from the transgenes. **a**, PTGS efficiency measured for potato virus (PVY) and Δ12-desaturase genes as the percentage of independent transgenic plants immune to PVY and the percentage of plants with enzyme activity reduced by more than 20% compared with wild type, respectively. In the predicted structures of RNA transcripts, right- and left-pointing arrows represent sense and antisense orientation of sequences, respectively; small vertical arrows represent splice-junction sequences remaining after the intron has been spliced out. Vertical lines in the predicted structures indicate duplex formation. Asterisks, data from ref. 3; daggers, data from ref 7; hpRNA, hairpin RNA; *n*, number of independent transformants; GUS, β-glucuronidase. **b**, Design of intron-containing hairpin constructs. OCS, octopine synthase; NOS, nopaline synthase.

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1. Hamilton, A. J., Lycett, G. W. & Grierson, D. *Nature* 346, 284–287 (1990).
2. Jorgensen, R. A. *Science* 268, 686–691 (1995).
3. Waterhouse, P. M., Graham, M. W. & Wang, M.-B. *Proc. Natl Acad. Sci. USA* 95, 13959–13964 (1998).
4. Waterhouse, P. M., Smith, N. A. & Wang, M.-B. *Trends Plant Sci.* 4, 452–457 (1999).
5. Wang, M.-B., Upadhyaya, N. M., Bretell, R. I. S. & Waterhouse, P. M. *J. Genet. Breed.* 51, 325–334 (1997).
6. Okuley, J. *et al. Plant Cell* 6, 147–158 (1994).
7. Cartea, M. E., Migdal, M., Gille, A. M., Pelletier, G. & Guercha, P. *Plant Sci.* 134, 161–169 (1998).
8. Rosche, E. & Westhoff, P. *Plant Mol. Biol.* 29, 663–678 (1995).
9. Gleave, A. P. *Plant Mol. Biol.* 20, 1203–1207 (1992).
10. Stalberg, K., Ellerstrom, M., Josefsson, L. G. & Rask, L. *Plant Mol. Biol.* 23, 671–683 (1993).
11. Fire, A. *et al. Nature* 391, 806–811 (1998).
12. Sharp, P. A. *Genes Dev.* 13, 139–141 (1999).

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